(PLG) complex has been generated based on our PGK structure and the reported three-dimensional structures of kringles (Fig.2).

The analysis of the PGK structure in complex with Kringle 2 from PLG suggested that other proteins containing kringle structures, such as tPA and uPA, could also formed bind PGK. PGK binding to tPA was confirmed experimentally (see communication by Simone Bergman) but not to uPA. Structural analysis based on PGK and kringle structures explains this preferential binding.

3. Conclusions

- Crystal structure of pneumococcal PGK has been solved at high resolution by X-ray crystallography.
- Structural analysis allowed us to locate the PLG-binding site at the N-terminal part of PGK. We determined how is the interaction between PGK and PLG.
- Computational model of PGK:K2 (PLG) complex suggested a potential interaction with plasminogen activator containing kringles.
- PGK can bind tPA but not uPA, structural analysis revealed that suitable changes in the lysine-binding sites of kringle domains may result into loss of binding.

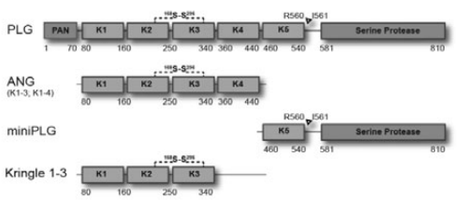

Fig. 1. Schematic model of PLG domain structure and used PLG derivatives. K, Kringle; PAN, PAN-apple (Pap)-domain; ANG, Angiostatin, miniPLG, mini-plasminogen.

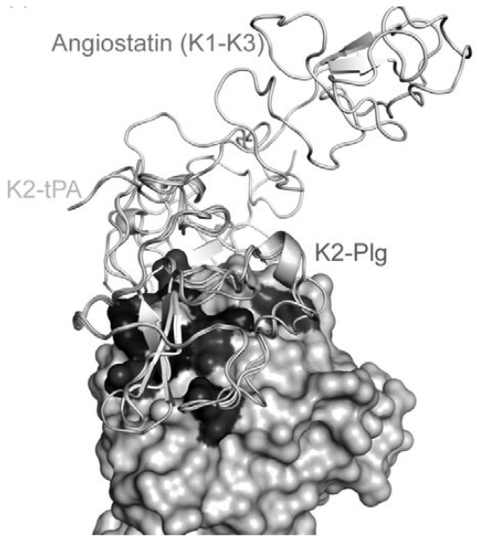

Fig. 2: Binding of PGK to K2-PLG, Angiostatin and k2-tPA.

CW_7 cell wall-binding motifs of the Cpl-7 endolysin target the peptidoglycan muropeptide

Noemí BUSTAMANTE [1,2], *Guadalupe GARCÍA* [1,2], *Roberto DÍEZ-MARTÍNEZ* [2,3], *Pedro GARCÍA* [2,3], *Marta BRUIX* [1], *Margarita MENÉNDEZ* [1,2]

1. Department of Biological Physical-Chemistry, Rocasolano Institute of Physical-Chemistry, CSIC, Serrano 119, 28006 Madrid, Spain
2. Ciber of Respiratory Diseases (CIBERES).
3. Department of Molecular Biology and Infection Biology, Center of Biological Research (CIB), CSIC, Ramiro de Maeztu 9, 28040 Madrid, Spain.

Contact name: Noemí Bustamante (n.bustamante@iqfr.csic.es)

ABSTRACT:

Phage endolysins are a novel class of efficient antimicrobials (enzybiotics) by their capacity to cleave the peptidoglycan of Gram-positive bacteria in a generally species-specific manner. The Cpl-7 endolysin, a lysozyme encoded by the Cp-7 bacteriophage, is a remarkable exception among all the murein hydrolases produced by *Streptococcus pneumoniae* and its bacteriophages, as it degrades pneumococcal cell walls containing either choline or ethanolamine. This behavior results from the acquisition of a C-terminal module made of three identical repeats of 42 amino acid each – the CW_7 motifs – specifically involved in cell wall attachment. Preliminary investigations were indicative that CW_7 repeats recognize the peptidoglycan network as target, with the potential impact of this fact on Cpl-7 antimicrobial host range. We have proved now, using STD-NMR spectroscopy, that N-acetyl-D-glycosaminyl-(β1,4)-N-acetylmuramyl-L-alanyl-D-isoglutamine, a structural analogue of the peptidoglycan monomer, is recognized by the CW_7 repeats and the contacts provided by this ligand have been identified. This finding could also explain the identification of CW_7 motifs in proteins involved in the cell wall metabolism that are encoded by Gram-positive and Gram-negative bacteria, including several pathogens, and by bacteriophages infecting Gram-positive bacteria.

Keywords: *Streptococcus pneumoniae*, enzybiotics, Cpl-7 endolysin, cell-wall targeting, STD-NMR.

1. Introduction

Cell wall lytic enzymes encoded by bacteriophages and bacteria constitute a novel and promising class of antimicrobials (enzybiotics). They usually consist of at least one catalytic module linked to other modules commonly involved in cell wall attachment and also responsible for their stringent substrate specificity [1, 2]. In the pneumococcal lytic enzymes this function relies on choline-binding modules (CBMs) that specifically recognize the choline moieties attached to teichoic and lipoteichoic acids, with the single exception of Cpl-7, a lysozyme encoded by the phage Cp-7. Cpl-7 comprises a catalytic module belonging to the family GH25 of glycosyl hydrolases and a C-terminal module made of three identical CW_7 repeats involved in cell wall attachment [3, 4]. This module is essential for Cpl-7 activity and accounts for its capacity of degrading pneumococcal cell walls containing either choline or ethanolamine. Thermal stabilization assays performed in the presence of small cell wall structural-analogues pointed to the cell wall muropeptide as the target recognized the CW_7 repeats, but direct evidences of complex formation were required [5].

This communication presents the characterization of the complexes formed by Cpl-7 full-length and its two isolated domains with N-Acetyl-D-

glycosaminyl-(β1,4)-N-acetyl muramyl-L-alanyl-D-isoglutamine, an analogue of the peptidoglycan monomer, using STD-NMR spectroscopy. Current results confirm the implication of the cell wall muropeptide in Cpl-7 attachment to the bacterial envelope and provide information on the ligand epitopes. Interestingly, the β anomer of the MurNAc moiety, the form present in the peptidoglycan, seems to be preferentially recognized with respect to the α anomer.

2. Experimental Section

2.1. Proteins and muropeptides

The Cpl-7 endolysin was produced and purified as previously described [4]. The His-tagged gene encoding the Cpl-7 N-terminal catalytic domain (N-Cpl-7) was built by overlap extension [6]. Appropriate nucleotides were used to clone the selected region and the PCR-amplifications into plasmid pQE3, with BamHI and KpnI as restrictions enzymes (5' and 3' ends, respectively). N-Cpl-7, expressed in *Escherichia coli* DH10B strain, was purified by Ni^{2+}-affinity chromatography. The gene fragment encoding the C-terminal module of Cpl-7 (C-Cpl-7) was synthesized in a pUC derivative plasmid, and subcloned in plasmid pT7-7 for expression in *E. coli* BL21(DE3). C-Cpl-7 was purified in two chromatographic steps (ion exchange and size exclusion chromatographies). Protein purity was checked by SDS-PAGE and mass spectrometry (MALDI_TOF) and concentrations measured using molar the extinction coefficients at 280-nm. *N*-Acetylmuramyl-L-alanyl-D-isoglutamine (Mur NAc-L-Ala-D-iso Gln) and N-Acetyl-D-glycosaminyl-(β1,4)-N-acetylmuramyl-L-alanyl -D-isoglutamine (GlcNAc-MurNAc-L-Ala-D-iso Gln) were from Sigma and CALBIOCHEM respectively. NMR samples (500 µL) were prepared in 20 mM phosphate buffer in D_2O, pH = 6.0, at protein concentrations of ca. 40 µM and ligand/protein ratios of 50:1.

2.2. Methods

NMR spectroscopy experiments were performed at 25 ºC in a Bruker Avance 800 MHz spectrometer equipped with inverse triple-resonance TCI cryo-probe and pulse gradients. The assignment of the 1H NMR spectra of the muropeptides was done on the bases of 2D spectra COSY, TOCSY (50 ms mixing time) and ROESY (125 ms mixing time) following standard procedures. Saturation-transfer difference NMR experiments (STD-NMR) were optimized using pulse sequences reported in literature [7, 8]. Spectra were recorded using a train of 50 ms Gaussian shaped pulses with flipping angles of 650º (height 87.7 Hz), 2.0 s of total irradiation time and 120 ms filtering trim pulse. The on-resonance frequency was set to -1.0 ppm, whereas off-resonance frequency was -150 ppm. Blank experiments were performed to assure the absence of direct saturation to the ligand protons. STD-TOCSY experiments were acquired at 800 MHz using 256 increments and an isotropic mixing time of 60 ms.

3. Results and Discussion

In STD-NMR spectroscopy, protein-ligand binding events lead to magnetization transfer from protons of the large receptor (full-length Cpl-7 and its isolated modules in this case) to the protons of the corresponding ligand. Thus, the protons of small molecules in close contact with the receptor in the bound state yield intense signal in the final difference spectrum, while remote protons show lower or null STD intensities. In this way the analysis of proton signals and intensities in STD-NMR allows the identification of the ligand-binding epitope in a quite accurate manner, if the NMR spectrum of the free ligand is already assigned. STD-NMR was therefore employed to detect muropeptide binding to the Cpl-7 endolysin and its two isolated modules, and the NMR spectra of the free ligands were recorded and fully assigned.

3.1. Assignment of NMR spectra of free muropeptides

In solution, MurNAc-L-Ala-D-isoGln and GlcNAc-MurNAc-L-Ala-D-isoGln exist as equilibrium mixtures of the α and β anomeric forms (ca. 2:1) at the reducing end of MurNAc [9]. Complete 1H assignment of both anomers in D2O was now achieved relying on standard 2D 1H-1H NMR experiments. The doublet signals of the anomeric protons were used as starting points in the assignment of sugar moieties of both anomers and, when possible, the 2:1 signal ratio was used to identify the corresponding peptide protons. Cross peak patterns of the TOCSY maps and sequential NOE correlations were also used to this aim. Two sets of signals were observed for all protons except for the GlcNAc moiety, showing that the anomeric equilibrium affects also to the environment of the peptide chain (see Table 1).

Table 1. ^{1}H chemical shift data (ppm) of GlcNAc-MurNAc-L-Ala-D-isoGln at 25ºC in Pi buffer (D_2O), pH 6.

Proton	^{1}α-anomer	β-anomer
GlcNAc-1	4.47	4.47
GlcNAc-2	3.68	3.68
GlcNAc-3	3.49	3.49
GlcNAc-4	3.35	3.35
GlcNAc-5	3.36	3.36
GlcNAc-6	3.70	3.70
GlcNAc-6'	3.87	3.87
GlcNAc-(CH3)	1.98	1.98
GlcNAc-(NH)	8.34	8.34
MurNAc-1	5.16	4.57
MurNAc-2	3.77	3.65
MurNAc-3	3.79	3.54
MurNAc-4	3.69	3.79
MurNAc-5	n.a.	3.41
MurNAc-6	3.65	3.62
MurNAc-6'	3.75	3.82
MurNAc-(CH_3)	1.89	1.87
MurNAc-(NH)	7.89	7.67
D-Lac- α	4.49	4.36
D-Lac- β	1.32	1.30
L-Ala-NH	8.36	8.24
L-Ala- α	4.24	4.23
L-Ala- β	1.38	1.38
D-iGln-NH	8.49	8.54
D-iGln- α	4.26	4.28
D-iGln-NH_2	7.61, 7.04	7.66, 7.07
D-iGln- β	2.04	2-04
D-iGln- β'	1.86	1.87
D-iGln-γ,γ'	2.24	2.24
1α and β refer to the anomeric form at the reducing MurNAC end		

3.2. STD-NMR detection of muropeptide targeting by full-length Cpl-7 endolysin and its isolated modules

The interaction of GlcNAc-MurNAc-L-Ala-D-isoGln with Cpl-7 and C-Cpl-7 was tested and proved by STD-NMR (*Fig. 1*) and the binding epitopes identified. Interestingly, the β anomer of MurNAc, the form present in the peptidoglycan, seems to be preferentially recognized with respect to the α anomer of the disaccharide-di-peptide. Ligand-provided contacts occur through several positions of the two saccharides units and the dipeptide. However, the strongest signals corresponded to the lactil group of MurNAc, and the methyl groups of MurNAc, GlcNAc and L-Ala. Recognition of GlcNAc was found to be determinant for the complex stability, as STD signals almost disappears in the presence of MurNAc-L-Ala-D-isoGln, in agreement with previous results [5]. Whether the CW_7/muropeptide interactions expand to other residues of the stem peptide or to the interpeptide bridges have yet to be elucidated. The differences in the relative intensities of the STD signals recorded with Cpl-7 and C-Cpl-7 suggested certain variations in their respective recognition of GlcNAc-MurNAc-L-Ala-D-isoGln. Such disparities might be due to the dual interaction of the disaccharide-di-peptide with the CW_7 repeats and with the catalytic module in the full-length protein. Indeed a similar but distinctive STD spectrum of GlcNAc-MurNAc-L-Ala-D-isoGln was observed in the presence of N-Cpl-7.

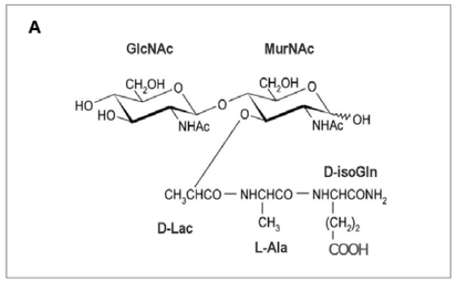

Fig. 1. (A) Scheme of GlcNAc-MurNAc-L-Ala-D-isoGln composition. (B) STD-NMR spectra of this compound in the presence of Cpl-7 full-length and the isolated cell wall-binding module(C-Cpl-7).

4. Conclusions

STD-NMR spectroscopy together with production of the isolated modules of Cpl-7 endolysin have allowed to identify the cell wall muropeptide as the structural motif targeted by the CW_7 repeats that form the cell wall-binding module of this endolysin.

The binding specificity of CW_7 repeats broadens the potential antimicrobial host range of Cpl-7 to other Gram-positive pathogens and even, under appropriate conditions, to Gram-negative bacteria.

Acknowledgements

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Fine tuning of pneumococcal cell division and elongation by the StkP kinase

Aurore FLEURIE [1], Chao ZHAO [1], Sylvie MANUSE [1], Nathalie CAMPO [2], Caroline CLUZEL [3], Jean-Pierre LAVERGNE [1], Céline FRETON [1], Christophe COMBET [1], Sébastien GUIRAL [1], Boumediene SOUFI [4], Boris MACEK [4], Anne-Marie DI GUILMI [5], Jean-Pierre CLAVERYS [2], Anne GALINIER [6] and Christophe GRANGEASSE [1]

1. BMSSI, Université Lyon 1, CNRS, 69367 Lyon, France.
2. LMGM, Université de Toulouse, CNRS, 31000 Toulouse, France.
3. LBTI, Université Lyon 1, CNRS, 69367 Lyon, France.
4. Proteome Center, University of Tuebingen, 72076 Tuebingen, Germany.
5. IBS, Université Joseph Fourier, CNRS, 38027 Grenoble, France.
6. LCB, Aix-Marseille Université, CNRS, 13402 Marseille, France.

Contact name: Grangeasse Christophe (c.grangeasse@ibcp.fr)

ABSTRACT:

To satisfy the different modes of cell division and the diversity of cell shapes, bacteria have developed species-specific mechanisms. Here, we report that the eukaryotic-like kinase StkP, a newly identified modulator of StkP kinase activity and one of the StkP endogenous phosphorylation targets (DivIVA) work together with FtsZ to achieve proper cell shape and division of *Streptococcus pneumoniae*. We conclude that the StkP/DivIVA/modulator triad orchestrates pneumococcal cell division and cell elongation and could constitute a widespread tool governing bacterial morphogenesis.

Keywords: protein phosphorylation, kinase, cell division, morphogenesis.

1. Introduction

Bacteria possess a versatile repertoire of protein-kinases [1]. We have demonstrated that the membrane Hanks-type serine/threonine kinase StkP plays a central role in regulating cell wall synthesis and controls septum positioning, assembly and closure in *Streptococcus pneumoniae* [2]. However, the underlying regulatory mechanisms remain unknown and investigating StkP-mediated phosphorylation represents a promising avenue to decipher the regulatory mechanisms governing S. pneumoniae morphogenesis and division.

2. Results

Here I will present our most recent results related to the role of StkP in pneumococcal cell morphogenesis. DivIVA could be phosphorylated by StkP and expression of non-phosphorylatable DivIVA affect cell shape integrity. We have also identified a modulator of StkP kinase activity that is required for StkP septal localization and subsequent phosphorylation of DivIVA. We show that the StkP/DivIVA/modulator triad connect the Z-ring to the peptidoglycan biosynthesis machinery and modulates the localization of some PBPs. In the light of these observations, we propose that StkP regulates the dynamics of septal and peripheral peptidoglycan synthesis responsible for the ovoid-shape of pneumococcus cells.

3. Conclusion

Many proteins involved in cytokinesis and morphogenesis are found to be phosphorylated in bacterial phospho-proteomes. We are thus only beginning to uncover the components of the phosphorylation toolkit regulating cell division.